FEBS Letters 467 (2000) 348–352 FEBS 23281

A family of ubiquitin-like proteins binds the ATPase domain of Hsp70-like Stch

Frederic J. Kaye[a,*], Sanjay Modi[a], Irena Ivanovska[b], Eugene V. Koonin[c], Kenneth Thress[d], Akihito Kubo[a], Sally Kornbluth[d], Mark D. Rose[b]

[a] *Medicine Branch, Division of Clinical Sciences, National Cancer Institute and National Naval Medical Center, Bethesda, MD 20889, USA*
[b] *Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA*
[c] *National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20814, USA*
[d] *Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC 27710, USA*

Received 17 December 1999

Edited by Horst Feldmann

Abstract We have isolated two human ubiquitin-like (UbL) proteins that bind to a short peptide within the ATPase domain of the Hsp70-like Stch protein. *Chap1* is a duplicated homologue of the yeast *Dsk2* gene that is required for transit through the G2/M phase of the cell cycle and expression of the human full-length cDNA restored viability and suppressed the G2/M arrest phenotype of *dsk2*Δ *rad23*Δ *Saccharomyces cerevisiae* mutants. *Chap2* is a homologue for *Xenopus scythe* which is an essential component of reaper-induced apoptosis in egg extracts. While the N-terminal UbL domains were not essential for Stch binding, Chap1/Dsk2 contains a Sti1-like repeat sequence that is required for binding to Stch and is also conserved in the Hsp70 binding proteins, Hip and p60/Sti1/Hop. These findings extend the association between Hsp70 members and genes encoding UbL sequences and suggest a broader role for the Hsp70-like ATPase family in regulating cell cycle and cell death events.

Key words: Cell cycle; Hsp70; Stch; Ubiquitin-like gene

1. Introduction

The *Hsp70*-like gene family encodes a group of related protein chaperones that are required for the viability of all living organisms. The structure of all Hsp70 proteins is similar and consists of a highly conserved 45 kDa N-terminal ATPase domain and a 25 kDa C-terminal domain that functions to reversibly capture nascent or denatured cellular polypeptides to initiate a wide range of protein processing events [1–3]. The complexity of Hsp70 activity has become increasingly apparent with the recognition that they interact with other co-chaperones, including Hsp40, Hsp90, and p60/Sti1, to form a functional unit [4]. In addition, non-chaperone 'Hsp70 interacting proteins' have been identified which are required for regulating protein folding and/or ATPase activity [5–11].

In contrast to the bi-functional structure of Hsp70 family members, a novel gene product was isolated, designated Stch, which encodes the 'core ATPase' domain of Hsp70 but lacks the peptide binding domain [12]. The truncated structure of Stch is conserved in *Caenorhabditis elegans*, rat, and human tissues where it was observed to resemble a proteolytically cleaved N-terminal ATPase fragment of Hsc70/Hsp70 [13]. To study the role of the Stch product in regulating protein processing, we performed a two-hybrid screen. We isolated multiple, overlapping human cDNA clones that encode distinct, ubiquitin-related proteins that bound efficiently to a conserved 20 amino acid region within the Stch ATPase domain. Analysis of the *Chap1*/*Dsk2* gene showed that it is a homologue of the *Saccharomyces cerevisiae DSK2* gene, which, together with *RAD23*, is important for the proper organization of the yeast mitotic spindle and transit through mitosis [14]. In contrast, *Chap2* represents the human *Bat3* gene [15] and is a homologue of the *Xenopus scythe* gene which is essential for reaper-induced apoptosis [16]. The identification of ubiquitin-linked proteins that bind to a conserved peptide motif within the 'core ATPase' Stch molecule suggests a broader role for Hsp70 family members in regulating specialized cellular events.

2. Materials and methods

2.1. Isolation of Stch binding proteins

Human *Stch* cDNA (codons 2–467) was subcloned in-frame into the pGBT9 Gal4p DNA binding domain plasmid (Clontech, Palo Alto, CA, USA). The HF7c yeast strain was transformed with the pGBT9-Stch plasmid followed by sequential transformation of a human lung cDNA library fused to the pGad10 Gal4p activation domain plasmid. Plasmids were isolated from yeast transformants on -L/W/H plates and subjected to nucleotide sequencing. Yeast strains were re-transformed with purified plasmids and multiple independent transformants were tested to confirm protein binding by β-galactosidase enzyme activity and by growth on SC-His media in all cases.

2.2. Functional analyses of Chap1/Dsk2

Yeast media and general techniques were as described previously [17]. Wild-type *S. cerevisiae* strain, MY3492, and the *dsk2*Δ *rad23*Δ mutant strain, MY5156, were transformed with: pMR3429, *pGAL* vector alone; pMR2757 *DSK2* CEN [14]; pMR4647 (*pGAL*-human Chap1/Dsk2); pMR2905, *pGAL-DSK2*; and pMR2906, *pGAL-DSK2-1*. Serial dilutions of transformants were incubated at the permissive or restrictive temperature and in the presence or absence of galactose and scored for growth [17]. Cultures of yeast transformants were also grown in SC-ura galactose medium until early logarithmic phase at 30°C and were shifted to 37°C for 10 h. Cells were fixed with methanol:acetone (3:1 ratio) on ice for 30 min and stained with DAPI on ice for 30 min. Greater than 100 cells were counted for each culture.

2.3. GST-Stch pull-down analysis with endogenous Bat3/Scythe

Glutathione-S-transferase (GST) and GST-fusion proteins were purified in *Escherichia coli* on glutathione-Sepharose beads as previously described [18], blocked with 10 μg/ml bovine serum albumin for 30 min, and washed three times in egg lysis buffer (ELB: 250 mM

*Corresponding author. NCI-Navy Oncology, Bldg 8/Rm 5101, Naval Hospital, Bethesda, MD 20889, USA. Fax: (1)-301-402 2414. E-mail: fkaye@helix.nih.gov

PII: S0014-5793(00)01135-2

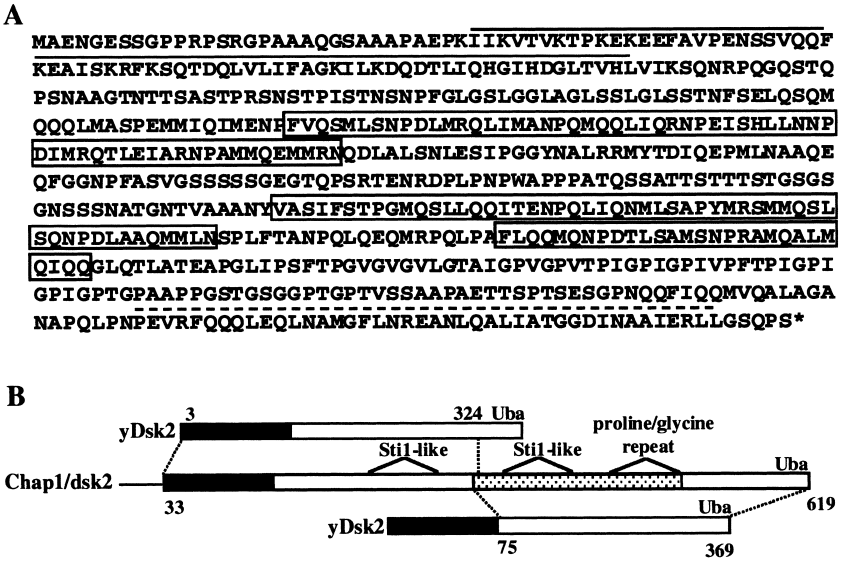

Fig. 1. A: Amino acid sequence of the human *Chap1/Dsk2* gene. The conserved ubiquitin domain (from residues 33 to 101) is overlined and is preceded by a 21 residue leader peptide; the conserved UbA domain is overlined by a broken line; the repeats resembling the C-terminal region of *S. cerevisiae* Sti1 and rat Hip are indicated by an open rectangle. B: Diagram of the alignment with the yeast *DSK2* gene (yDsk2) showing duplication of the human homologue. The ubiquitin domain is depicted as a black rectangle, the UbA domain (aa 326–369 in yDsk2; aa 578–620 in Chap1/Dsk2) is identified, and the minimal sequence for Stch binding (aa 319–520) is shown as a stippled rectangle. GenBank accession number is AF189009.

sucrose, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 10 mM HEPES pH 7.4). A crude *Xenopus* egg extract was prepared in ELB as previously described [16] and added to the GST-fusion proteins at 10× the bead volume. Following a 60 min incubation at 4°C, the GST-fusion proteins were pelleted and washed three times in ELB. The washed pellets were resolved by SDS–PAGE and subjected to protein immunoblotting using 1:500 dilution of an α-Scythe antisera raised against the carboxy-terminal 16 amino acids of the *X. scythe* protein.

2.4. Protein sequence analysis

The non-redundant database of protein sequences at the National Center for Biotechnology Information (NCBI, NIH, Bethesda, MD, USA) was tested using the gapped BLASTP program and the Position-Specific Iterating (PSI) BLAST program [19]. Conserved signaling and interaction domains in protein sequences were identified using the SMART searching engine [20]. Multiple sequence alignments were constructed using the Clustal W program [21]. The accession numbers for the amino acid sequences used: *S. cerevisiae DSK2* (P48510), *S. cerevisiae SSA4* (P22202), human Stch (U04735), and human BiP (P11021).

3. Results and discussion

We obtained several HF7c His+ transformants after screening a human lung two-hybrid cDNA library using a pGBT9-Stch bait plasmid. Three overlapping clones were isolated for

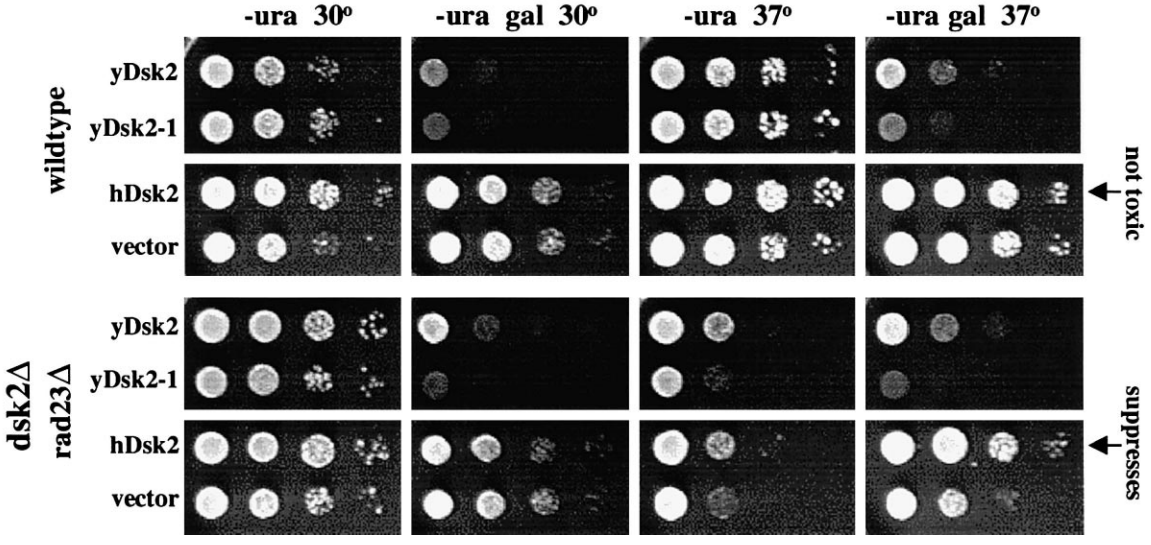

Fig. 2. The human *Chap1/Dsk2* gene suppressed the *dsk2*Δ *rad23*Δ phenotype. Serial dilutions of wild-type or temperature-sensitive *dsk2*Δ *rad23*Δ mutant yeast colonies were grown at the permissive or restrictive temperatures as indicated. The yeast colonies contained either wild-type yeast *DSK2* (yDSK2), dominant mutant yeast *DSK2* (yDSK2-1), the human *Chap1/Dsk2* (hDsk2), or the empty vector. All of the *DSK2* genes were expressed as galactose-inducible proteins.

plasmid				
vector	15	11	**73**	1
yDsk2	36	23	**30**	11
hDsk2	25	21	**46**	8

Fig. 3. The human *Chap1*/*Dsk2* gene suppresses the G2/M arrest phenotype of *dsk2*Δ *rad23*Δ. Strain MY5156 (*dsk2*Δ *rad23*Δ) was transformed with the indicated plasmids as described in Section 2. Cell types shown are (left to right): unbudded G1 phase cells, small-budded S/G2 phase cells, large-budded G2/M phase cells, and large-budded post-mitotic cells.

the *Chap1* gene, one of which represented a full-length cDNA spanning 3360 nucleotides and encoding an open reading frame of 624 residues (Fig. 1). A Blastp search [22] revealed that this gene represented the human homologue for the yeast *DSK2* gene [14]. Inspection of the amino acid alignment between the human and yeast homologues shows that the human gene encodes a 21 residue leader peptide, resembling a mitochondrial import sequence, followed by a conserved 70 amino acid, ubiquitin-like (UbL) domain at the amino-terminal end. Chap1/Dsk2, however, is approximately twice the size of the yeast homologue, apparently resulting from a duplication of the yeast sequence distal to the UbL domain. In addition, both the yeast and human genes contain a ubiquitin-associated (UbA) domain at their C-termini [23].

Neither the N-terminal UbL domain nor the C-terminal UbA domain of Chap1/Dsk2 were required for binding to Stch and the smallest cDNA clone isolated in our screen spanned 200 residues of one monomer *DSK2* unit suggesting that the duplication of the yeast sequence was not essential for Stch binding. Interestingly, this minimal binding domain contained two Sti1-like repeat sequences with strong sequence similarity to the conserved C-terminal domain of the chaperone binding proteins p60/Sti1 and Hip [24] (Fig. 1) (Chap1 residues: 190–237, 360 to 412, and 433–460 align with the *S. cerevisiae* Sti1 residues: 149–191 and 540 to 579) that in Sti11 is implicated in Hsp70 and Hsp90 binding [24,25]. We observed that an in-frame deletion of Chap1/Dsk2 removing amino acid residues 418 to 431 that lie between the last two Sti1 repeat sequences within the minimal binding region had no effect on Stch binding using the yeast two-hybrid binding assay. In contrast, a carboxy-terminal deletion that disrupted only the final Sti1 repeat sequence showed complete loss of Stch binding (data not shown) suggesting that this region is required for binding to the ATPase domain of Stch. In addition, the minimal binding domain of Chap1/Dsk2 spans a 55 residue glycine/proline collagen-like repeat region which resembles, but is distinct, from the GGMP repeat observed in the Hip product [24].

To examine the functional activity of Chap1/Dsk2, a temperature-sensitive *dsk*2Δ *rad23*Δ *S. cerevisiae* strain (MY5156) was tested for cell growth following transformation with the full-length yeast *DSK2* gene (yDsk2), a dominant yeast *DSK2* mutation in the ubiquitin domain (*yDSK2-1*), the human *Chap1*/*Dsk2* cDNA (hDsk2), or the vector alone (Fig. 2). All of the proteins were expressed under the control of the *GAL1* galactose-inducible promoter. While single mutants for either *dsk2*Δ or *rad23*Δ do not exhibit cell growth defects at 37°C, double mutants at the restrictive temperature are arrested at G2/M with defects in duplication of the mitotic spindle pole body [14]. We observed that high levels of *yDSK2* and *yDSK2-1* expression were toxic in wild-type *S. cerevisiae* cells [14]. However, as reported previously, when expressed under its own promoter, *yDSK2* could reverse the block at G2/M observed in *dsk2*Δ *rad23*Δ cells (Fig. 3 and data not shown) [14]. Following galactose induction, the human *Chap1*/*Dsk2* gene efficiently suppressed the growth arrest of the *dsk2*Δ *rad23*Δ cells, and, in contrast to yeast *DSK2*, high level expression of the human homologue was not toxic to wild-type cells. In addition, we analyzed the cell cycle distribution of the *dsk2*Δ *rad23*Δ transformants following 10 h incubation at the restrictive temperature. We observed a reduction in the frequency of 'aberrant' large-budded cells at G2/M in cells transformed with the human Chap1/Dsk2 cDNA clone, confirming the ability of the human homologue to complement the *dsk2*Δ *rad23*Δ defect (Fig. 3). These results indicate that the sequence conservation between the yeast and human proteins extends to functional conservation.

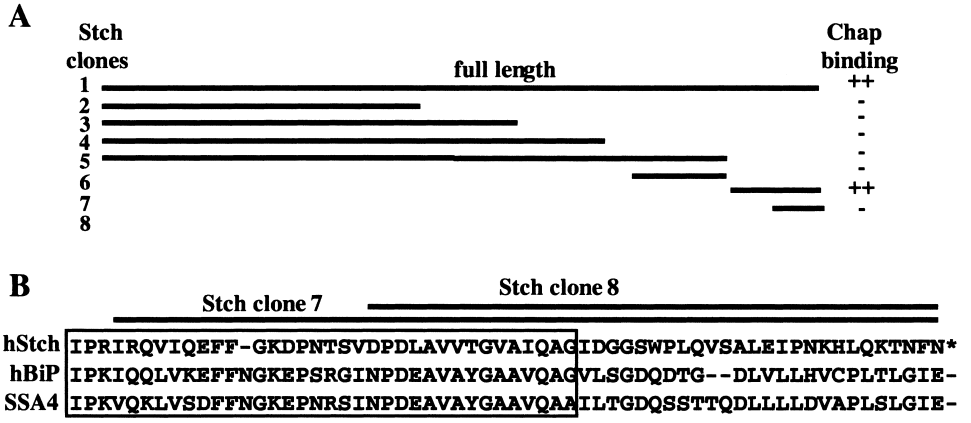

Fig. 4. Chap1/Dsk2 binds to a conserved peptide at the carboxy-terminal end of the ATPase domain. A: Diagram of Stch peptide clones. ++ indicates strongly positive β-galactosidase activity and abundant growth on SC-His media. − indicates absent β-galactosidase activity and no growth on SC-His plates after 4 days of incubation. B: Partial amino acid sequence alignment showing human Stch (hStch), human BiP/Grp78 (hBip), and the *S. cerevisiae* Hsp70 product *SSA4*. The terminal, conserved region of the Hsp70-like ATPase domain is boxed with the corresponding Stch clones overlined.

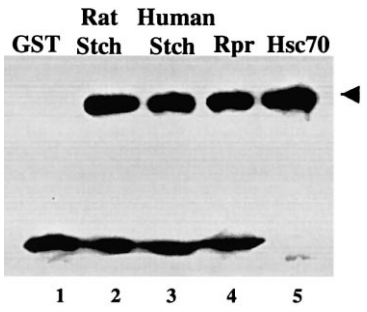

Fig. 5. Immunoblot analysis showing GST-Stch, GST-Hsc70, and GST-reaper (rpr) bind endogenous Scythe. Recombinant GST protein alone or the indicated GST-fusion proteins were incubated in the presence of a *Xenopus* egg extract as described in Section 2 and the washed pellets were resolved by SDS–PAGE and subjected to protein immunoblot analysis with α-Scythe antisera.

To test the specificity of the binding interaction with the ATPase domain of Stch, we used the two-hybrid technique to map the binding site for Chap1/Dsk2 on the Stch protein (Fig. 4). Using both β-galactosidase activity and growth on SC-His plates, we observed that a series of carboxy-terminal Stch truncations showed loss of protein binding. Since Stch contains a unique carboxy-terminal sequence that is conserved in all Stch homologues, but not shared with other Hsp70 members [13], we designed two peptide sequences that would either include the C-terminal 30 amino acid residues that are conserved in all Hsp70 members (clone 7) or would include unique Stch sequences (clone 8). We observed that clone 7 bound Chap1/Dsk2 with the same efficiency as full-length Stch, while the smaller peptide containing Stch-specific, C-terminal sequences had no binding activity. Therefore, Stch binds Chap1/Dsk2 via a region that is highly conserved in Hsp70 proteins.

The interaction of Chap1/Dsk2 with the ATPase domain of Stch resembles the properties of the Bag1/Rap46 protein which also contains an N-terminal UbL domain and was recently shown to bind to the ATPase domain of 70 kDa heat shock proteins [5,11]. We have also isolated multiple cDNA clones for another UbL containing gene, designated *Chap2*, that bound efficiently to pGBT9-Stch in the two-hybrid screen. Chap2 represented the human homologue for the *Bat3*/*Scythe* gene [15,16] and exhibits several similarities with Chap1/Dsk2 including the presence of an N-terminal UbL domain which was again not required for Stch binding and showed an identical pattern of binding exclusively to the short conserved motif localized on Stch ATPase peptide 7 (Fig. 4).

To confirm the Scythe-Stch interaction, we tested the binding of endogenous *X. scythe* to either GST alone, the previously reported Scythe ligand, GST-reaper [16], or to either human or rat GST-Stch (Fig. 5). We found that both rat and human Stch precipitated Scythe from *Xenopus* egg extracts with an efficiency similar to the pro-apoptosis *Drosophila* reaper product (GST-rpr), while the GST leader peptide had no Scythe binding activity. We also demonstrated binding of endogenous Scythe to full-length *Xenopus* Hsc70 suggesting a more general role for this binding interaction (Fig. 5). These observations demonstrate that the ATPase domain of Hsp70-like members binds to a family of at least three ubiquitin-linked proteins, including Chap1/Dsk2, Chap2/Scythe, and Bag1/Rap46.

The role for these interactions with UbL-linked proteins, however, is still unknown. The best-characterized UbL-linked protein is Rad23p, which controls UV sensitivity [26,27], regulates Rad4p activity, and directly interacts with the 26S proteasome through its amino-terminal ubiquitin domain [28]. Since Rad23p binds to the Rad4p DNA repair protein through its carboxy-terminal region [29], this protein represents a direct link between DNA repair and the ubiquitin/proteasome pathways [28]. While we have shown that Rad23 and Chap1/Dsk2 share conserved UbL and UbA domains [23,30] and are functionally redundant for the ability to complement cell growth in *dsk2*Δ and *rad23*Δ *S. cerevisiae* cells, there is no evidence to date that either Chap1/Dsk2, Scythe, or Bag1/Rap46 are associated with the proteasome. In the case of Bag1/Rap46, recent studies have shown that the binding of Bag1/Rap46 with Hsp/Hsc70 serves to both inhibit chaperone activity and to mediate multimeric protein complexes that include the anti-apoptosis molecule, Bcl-2, the nuclear oncogene, c-jun, as well as several hormone and growth factor receptors [5,9,11]. These observations suggested that Bag1/Rap46 may serve to regulate the cellular stress response and provide a link with cell signalling and the cell death machinery. Although we have not yet defined the subcellular localization and the specific functional role for Chap1/DSk2 and Chap2/Scythe, we have now identified two additional ubiquitin-linked proteins that retain the capacity to bind to the ATPase domain of an Hsp70-like molecule and which can modulate, respectively, transit through the G2/M phase of the cell cycle or the apoptotic machinery. Of interest, a recently identified Hsp70 interacting protein, CHIP [7] also encodes a discrete ubiquitin box domain (U-box) identified in a novel ubiquitin conjugation factor involved in multiubiquitin assembly and cell survival under stress conditions [31]. These findings propose a broader role for the Hsp70-like family in regulating cell cycle and cell death events.

Acknowledgements: This work was supported in part by a grant from the National Institutes of Health (to M.D.R.), an NJCCR fellowship (to I.I.), and an NIH grant: RO1 GM56518 (to S.K.). S.K. is a Scholar of the Leukemia Society of America. K.T. is a predoctoral fellow of the USARMC breast Cancer program. We thank Ahad Rehmatulla for technical assistance.

References

[1] Hartl, F.U. (1996) Nature 381, 571–580.
[2] Pelham, H.R. (1986) Cell 46, 959–961.
[3] Flynn, G.C., Pohl, J., Flocco, M.T. and Rothman, J.E. (1991) Nature 353, 726–730.
[4] Chang, H.C., Nathan, D.F. and Lindquist, S. (1997) Mol. Cell. Biol. 17, 318–325.
[5] Takayama, S., Bimston, D.N., Matsuzawa, S., Freeman, B.C., Aime-Sempe, C., Xie, Z., Morimoto, R.I. and Reed, J.C. (1997) EMBO J. 16, 4887–4896.
[6] Raynes, D.A. and Guerriero Jr., V. (1998) J. Biol. Chem. 273, 32883–32888.
[7] Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y. and Patterson, C. (1999) Mol. Cell. Biol. 19, 4535–4545.
[8] Jiang, R.F., Greener, T., Barouch, W., Greene, L. and Eisenberg, E. (1997) J. Biol. Chem. 272, 6141–6145.
[9] Hohfeld, J. and Jentsch, S. (1997) EMBO J. 16, 6209–6216.
[10] Takayama, S., Sato, T., Krajewski, S., Kochel, K., Irie, S., Millan, J.A. and Reed, J.C. (1995) Cell 80, 279–284.
[11] Zeiner, M., Gebauer, M. and Gehring, U. (1997) EMBO J. 16, 5483–5490.

[12] Otterson, G.A., Flynn, G.C., Kratzke, R.A., Coxon, A., Johnston, P.G. and Kaye, F.J. (1994) EMBO J. 13, 1216–1225.
[13] Otterson, G.A. and Kaye, F.J. (1997) Gene 199, 287–292.
[14] Biggins, S., Ivanovska, I. and Rose, M.D. (1996) J. Cell Biol. 133, 1331–1346.
[15] Banerji, J., Sands, J., Strominger, J.L. and Spies, T. (1990) Proc. Natl. Acad. Sci. USA 87, 2374–2378.
[16] Thress, K., Henzel, W., Shillinglaw, W. and Kornbluth, S. (1998) EMBO J. 17, 6135–6143.
[17] Rose, M.D., Winston, F. and Heiter, P. (1990), 198 pp. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–40.
[19] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[20] Ponting, C.P., Schultz, J., Milpetz, F. and Bork, P. (1999) Nucleic Acids Res. 27, 229–232.
[21] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[22] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) J. Mol. Biol. 215, 403–410.
[23] Hofmann, K. and Bucher, P. (1996) Trends Biochem. Sci. 21, 172–173.
[24] Hohfeld, J., Minami, Y. and Hartl, F.U. (1995) Cell 83, 589–598.
[25] Lassle, M., Blatch, G.L., Kundra, V., Takatori, T. and Zetter, B.R. (1997) J. Biol. Chem. 272, 1876–1884.
[26] Sugasawa, K., Masutani, C., Uchida, A., Maekawa, T., van der Spek, P.J., Bootsma, D., Hoeijmakers, J.H. and Hanaoka, F. (1996) Mol. Cell Biol. 16, 4852–4861.
[27] Watkins, J.F., Sung, P., Prakash, L. and Prakash, S. (1993) Mol. Cell. Biol. 13, 7757–7765.
[28] Schauber, C., Chen, L., Tongaonkar, P., Vega, I., Lambertson, D., Potts, W. and Madura, K. (1998) Nature 391, 715–718.
[29] Wang, Z., Wei, S., Reed, S.H., Wu, X., Svejstrup, J.Q., Feaver, W.J., Kornberg, R.D. and Friedberg, E.C. (1997) Mol. Cell. Biol. 17, 635–643.
[30] Dieckmann, T., Withers-Ward, E.S., Jarosinski, M.A., Liu, C.F., Chen, I.S. and Feigon, J. (1998) Nat. Struct. Biol. 5, 1042–1047.
[31] Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H.D., Mayer, T.U. and Jentsch, S. (1999) Cell 96, 635–644.